

Chronic adriamycin treatment impairs CGRP-mediated functions of meningeal sensory nerves

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ABSTRACT

Adriamycin is a potent anthracycline-type antitumor agent, but it also exerts potentially serious side effects due to its cardiotoxic and neurotoxic propensity. Multiple impairments in sensory nerve functions have been recently reported in various rat models. The present experiments were initiated in an attempt to reveal adriamycin-induced changes in sensory effector functions of chemosensitive meningeal afferents.

Meningeal blood flow was measured with laser Doppler flowmetry in the parietal dura mater of adult male Wistar rats. The dura mater was repeatedly stimulated by topical applications of capsaicin, a transient receptor potential vanilloid 1 (TRPV1) receptor agonist, or acrolein, a transient receptor potential ankyrin 1 (TRPA1) receptor agonist, which induce the release of calcitonin gene-related peptide (CGRP) from meningeal afferents. The blood flow increasing effects of CGRP, histamine, acetylcholine and forskolin were also measured. Capsaicin- and acrolein-induced CGRP release was measured with enzyme-linked immunoassay in an *ex vivo* dura mater preparation. TRPV1 content of trigeminal ganglia and TRPV1-, CGRP- and CGRP receptor component-immunoreactive structures were examined in dura mater samples obtained from control and adriamycin-treated rats.

The vasodilator effects of capsaicin, acrolein and CGRP were significantly reduced in adriamycin-treated animals while histamine-, acetylcholine- and forskolin-induced vasodilatation were unaffected. Measurements of CGRP release in an *ex vivo* dura mater preparation revealed an altered dynamic upon repeated stimulations of TRPV1 and TRPA1 receptors. In whole-mount dura mater preparations immunohistochemistry revealed altered CGRP receptor component protein (RCP)-immunoreactivity in adriamycin-treated animals, while CGRP receptor activity modifying protein (RAMP1)-, TRPV1- and CGRP-immunostaining were left apparently unaltered. Adriamycin-treatment slightly reduced TRPV1 protein content of trigeminal ganglia.

The present findings demonstrate that adriamycin-treatment alters the function of the trigeminovascular system leading to reduced meningeal sensory neurogenic vasodilatation that may affect the local regulatory and protective mechanisms of chemosensitive afferents leading to alterations in tissue integrity.

1. Introduction

Anthracyclines comprise an important class of chemotherapeutic agents still indispensable in the treatment of various malignancies (Carvalho et al., 2009; Kalyanaraman et al., 2002). Adriamycin, despite its potentially serious side effects, is one of the most commonly used anthracycline derivative for its favorable antineoplastic activity. Besides its deleterious effects on cardiac muscle resulting in congestive cardiomyopathy, adriamycin exerts neurotoxic effects on primary sensory neurons in experimental animals and also in man (Bigotte and Olsson, 1982; Katona et al., 2004; Kondo et al., 1987; Minow and Gottlieb,

1975). Recent studies demonstrated marked structural, neurochemical and functional impairments of primary sensory neurons in animal models of adriamycin toxicity (El-Agamy et al., 2017; Kosoko et al., 2017). Neurotoxic propensity of adriamycin manifests as deleterious actions on chemosensitive sensory neurons which express transient receptor potential nociceptive ion channels (Boros et al., 2016). The non-selective cation channels transient receptor potential vanilloid 1 (TRPV1) and transient receptor potential ankyrin 1 (TRPA1) are multimodal sensor proteins that sense temperature (cold or hot), the presence of exogenous or endogenous noxious chemicals and acidic pH (Nilius et al., 2012; Numazaki and Tominaga, 2004; Szallasi, 1994).

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Chemosensitive afferent neurons innervate most somatic and visceral organs and tissues (Jancsó et al., 1987) and express the TRPV1 (Nilius et al., 2012) and the TRPA1 (Bodkin and Brain, 2011; Zygmunt and Högestätt, 2014) receptors, which play crucial roles in nociceptive and neurogenic vascular and inflammatory reactions (Dux et al., 2003; Earley and Brayden, 2015; Eberhardt et al., 2014; Geppetti et al., 2008; Gouin et al., 2017). Activated chemosensitive afferent nerves transmit nociceptive signals towards the central nervous system and concomitantly release neuropeptides, such as calcitonin gene-related peptide (CGRP) and substance P (SP) from their peripheral terminals leading to arterial vasodilatation, increase in vascular permeability and degranulation of mast cells (Brain, 1997; Holzer, 1998; Jansen-Olesen et al., 2014; Maggi, 1995). Since significant proportions of TRPV1 and TRPA1 expressing chemosensitive sensory neurons are peptidergic (Aubdool and Brain, 2011), the demonstration of peptide mediated vascular reactions and the quantitative measurement of the release of CGRP are reliable functional indicators of the activation of this particular class of nociceptors.

Local regulatory functions induced by neuropeptides released from chemosensitive afferents contribute also to protective mechanisms in peripheral tissues (Kashem et al., 2015; Russell et al., 2014). Preclinical experiments indicate that CGRP released from cardiac chemosensitive afferents is protective against adriamycin-induced cardiomyopathy (Ferdinandy et al., 1997; Katona et al., 2004; Shi et al., 2011). Recent observations from our laboratory have revealed profound structural and functional changes in cutaneous chemosensitive nociceptors following systemic adriamycin treatment. Adriamycin impaired both the sensory afferent function of chemosensitive nociceptors and the local efferent functions mediated by the release of neuropeptides (Boros et al., 2016; Katona et al., 2004). Testing peripheral neurogenic vascular reactions seems to be a useful tool to predict possible risks of adriamycin-induced impairments of organ function. Since adriamycin-induced changes in sensory functions develop prior to the manifestation of its cardiotoxic effect, they may also signalize upcoming cardiac injury (Boros et al., 2016).

The mechanisms of adriamycin-induced pathophysiological changes affecting sensory and vascular functions are not fully understood. The susceptibility of the different compartments of the primary sensory neuron to adriamycin appears to be diverse, too; nociceptor endings seem to be more affected than the rest of the neuron. In the skin, adriamycin treatment decreases the density of epidermal nerve fibers while leaving the density and distribution of subepidermal nerve fibers apparently normal (Boros et al., 2016). Adriamycin seems to impair also the contractility of vascular smooth muscle, probably by oxidative stress modifying protein functions (Murata et al., 2001).

The present experiments were initiated in an attempt to reveal adriamycin-induced changes in vascular and sensory effector functions of dural chemosensitive afferent nerves in a well-established animal model of meningeal nociception. Morphological and functional traits of trigeminal afferents innervating meningeal tissues in rats have already been extensively studied in our laboratory (Dux et al., 2009, 2016a; Marics et al., 2016). These studies have revealed that a substantial proportion of dural afferent nerves are capsaicin-sensitive, contain CGRP and express the nociceptive ion channels TRPV1 and TRPA1 (Dux et al., 2017; Dux et al., 2016b). Therefore, in the present study changes in meningeal blood flow and CGRP release were measured in control and adriamycin-treated rats upon dural applications of specific agonists of TRPV1 and TRPA1 in an open cranial window model. Changes in TRPV1 protein content of trigeminal ganglia, expression and distribution of TRPV1 receptor immunoreactivity, CGRP immunoreactivity and immunoreactivity of vascular CGRP receptor components in the dura mater were also determined.

2. Methods

2.1. Animals

The experiments were approved by the Ethical Committee for Animal Care of the University of Szeged. Study procedures were carried out in accordance with the Directive 2010/63/EU of the European Parliament. All efforts were made to minimize the number of animals used and their suffering.

Adult male Wistar rats weighing 270–330 g were used. One group of animals received a cumulative dose of 15 mg/kg of adriamycin (Doxorubicin, Pharmacia Italia, Italy) by intravenous injection of 2.5 mg/kg of the drug three times a week for 2 weeks (Tong et al., 1991). Control rats received equivalent amounts of the vehicle (saline). All experiments were performed 2–7 days after cessation of the treatment.

2.2. In vivo measurement of meningeal blood flow

Control and adriamycin-treated animals were anesthetized with thiopental sodium (100 mg/kg, i.p. Insera Arzneimittel GmbH Freiburg, Germany). The animals were tracheotomized and breathed spontaneously. Systemic blood pressure was recorded with a pressure transducer connected to a cannula inserted into the femoral artery. The body temperature was monitored with a thermoprobe inserted into the rectum and was held at 37–37.5 °C by a heating pad. For the measurement of meningeal blood flow, a cranial window was prepared according to Kurosawa et al. (1995). The head of the animal was stabilized in a stereotaxic frame, the scalp was incised in the midline and the parietal bone was exposed on one side. A cranial window was drilled into the parietal bone to expose the underlying dura mater. Blood flow was recorded with a needle-type probe of a laser Doppler flowmeter (Perimed, Sweden) positioned over a branch of the middle meningeal artery. Data on meningeal blood flow and systemic blood pressure were processed with the Perisoft program (Perimed, Sweden). Stimulation of the dura mater was performed by repeated topical applications of capsaicin (100 nM), acrolein (300 µM) and CGRP (10 µM) at a volume of 40 µl for 3 min followed by repeated washouts with synthetic interstitial fluid (SIF, containing in mM: 135 NaCl, 5 KCl, 1 MgCl₂, 5 CaCl₂, 10 glucose and 10 Hepes, pH 7.4). During washout periods the basal blood flow was restored. The effects of single histamine (10 µM), acetylcholine (100 µM) and forskolin (100 µM) applications were also tested. All drugs were purchased from Sigma-Aldrich Chemie GmbH, Hungary. A stock solution of capsaicin (32 mM) was prepared with the aid of 6% ethanol and 8% Tween 80 in saline and was further diluted with SIF. All the other drugs were dissolved in SIF immediately before use. Basal blood flow was determined as the mean flow during a 3 min period prior to drug application. Percentage changes in meningeal blood flow in response to capsaicin, acrolein, CGRP, histamine, acetylcholine and forskolin were determined as mean flow values within the 3 min application period relative to the basal flow. At the end of the experiments, the animals were sacrificed by an overdose of thiopental sodium (200 mg/kg, i.p.).

2.3. Ex vivo measurement of CGRP release

Measurement of the release of CGRP from dural afferents was performed by the method of Ebersberger et al. (1999). Control and adriamycin-treated animals were deeply anesthetized with thiopental sodium (200 mg/kg, i.p.) and decapitated. After removal of the skin and muscles, the skull was divided into halves along the midline and the cerebral hemispheres were removed. The skull preparations were washed with carbogen-gassed SIF at room temperature for 30 min and then mounted in a humid chamber at 37 °C. The cranial fossae were filled with 300 µl of carbogen-gassed SIF solution. Samples of the perfusate were collected at periods of 5 min by carefully removing the

content of the skull halves with a pipette. Control samples were taken to determine basal CGRP release then the dura was stimulated with repeated applications of capsaicin at 100 nM or acrolein at 300 μ M. Capsaicin and acrolein were applied three times separated by washout periods with SIF. The CGRP releasing effect of KCl (60 mM) application was also measured and compared with basal CGRP release. 100 μ l of samples diluted with 25 μ l of enzyme-linked immunoassay (EIA) buffer were placed into Eppendorf cups and immediately frozen at -70°C for subsequent analysis. The EIA method was used for measurement of CGRP concentration (Bertin Pharma, France). The CGRP concentrations of the superfusates were expressed in pg/ml. Changes induced in CGRP release by capsaicin, acrolein and KCl were expressed as percentage changes relative to the basal release.

2.4. Measurement of TRPV1 protein content in the trigeminal ganglion

Control and adriamycin-treated animals were deeply anesthetized with thiopental sodium (200 mg/kg, i.p.) and decapitated. After removal of the skin and muscles, the skull was divided into halves along the midline. The trigeminal ganglia were cut out, homogenized in phosphate buffered saline and stored overnight at -20°C . Two freeze-thaw cycles were performed before centrifuging the homogenates at 4°C for 5 min at 5g. The supernatants were removed and frozen at -70°C for subsequent analysis. EIA method was used for the determination of TRPV1 protein in tissue samples (Aviva Systems Biology, USA). The TRPV1 concentration was expressed in pg/mg tissue.

2.5. Immunohistochemistry

The distribution of TRPV1- and CGRP-immunoreactive nerve fibers as well as CGRP receptor component protein- (RCP) and receptor activity modifying protein (RAMP1)-immunoreactivities were studied in dural whole mount preparations of control and adriamycin-treated rats not used for *in vivo* blood flow recordings or *ex vivo* CGRP release experiments previously. Animals were anesthetized deeply with thiopental sodium (200 mg/kg, i.p.) and perfused transcardially with physiological saline followed by 4% paraformaldehyde in phosphate buffer (pH 7.4). The skin and muscles of the skull were removed and the skull was divided into halves along the sagittal suture. After removing the brain, samples of the dura mater containing branches of the middle meningeal artery were cut out, postfixed for 2 h in the same fixative and processed for staining with the indirect immunofluorescence technique using a rabbit polyclonal antiserum raised against the TRPV1 receptor (1:500, Alomone Laboratories, Israel) in combination with a monoclonal mouse anti-CGRP antibody (1:500, Sigma-Aldrich, Germany). IgGs labeled with Cy3 and DyLight 488 were used as secondary antibodies (both 1:500, Jackson ImmunoResearch Laboratories, USA). CGRP receptor components RCP and RAMP1 were visualized using mouse antiserum against RCP in combination with goat anti-RAMP1 (both 1:50, Santa Cruz Biotechnology, USA) primary and corresponding secondary antibodies labeled with Alexa 555 and Alexa 488 (both 1:500, Molecular Probes, USA). Whole mount preparations of the dura mater were examined under a confocal fluorescence microscope (ZEISS LSM 700, Germany).

2.6. Statistics

All values were expressed as means \pm SEM. Statistical analysis of the data was performed using Statistica 13 (StatSoft, Tulsa, OK, USA). In all groups normality was tested by the Shapiro-Wilk test. According to the distribution of data the Student's *t*-test or the Wilcoxon test was used. ANOVA with repeated measurements and Fisher's least significant difference test were used to analyze the consecutive measurements of CGRP levels and blood flow increases induced by repeated applications of capsaicin, acrolein and CGRP. A probability level of $p < 0.05$ was regarded as statistically significant.

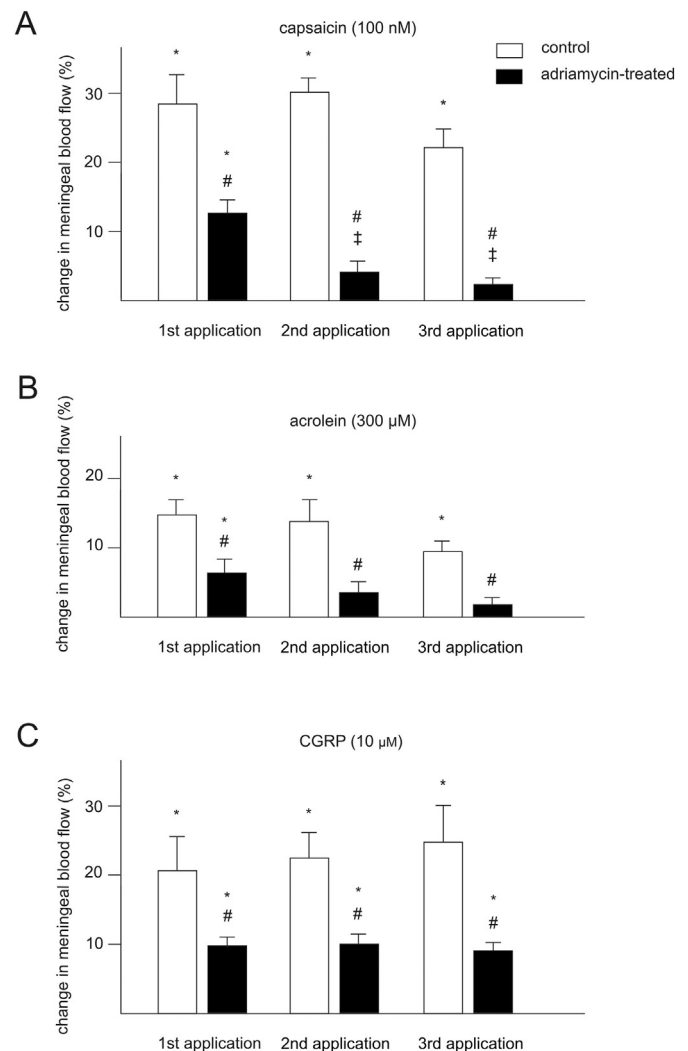


Fig. 1. Effect of repeated applications of capsaicin (100 nM, A) acrolein (300 μ M, B) and CGRP (10 μ M, C) on meningeal blood flow. *: statistically different from the basal flow, #: statistically different from the control, ‡: statistically different from the effect of the first application.

3. Results

3.1. Adriamycin-treatment impairs meningeal blood flow upon TRPV1 and TRPA1 receptor activation

In all experiments blood flow was recorded from topographically identical segments of the middle meningeal artery distant from visible cortical blood vessels. The basal flow values were between 200 and 500 perfusion units (PU) in control and adriamycin-treated rats. Topical application of capsaicin (100 nM) in control rats was followed by a significant increase ($28.6 \pm 7.9\%$, $n = 8$) in meningeal blood flow, while in adriamycin-treated animals this response was weaker ($11 \pm 3\%$, $n = 14$). Capsaicin-induced increases in meningeal blood flow were reproducible in control animals, separated by washout periods; the second and third applications of capsaicin increased meningeal blood flow by 30.3 ± 4 and $21.5 \pm 3.4\%$, respectively. In adriamycin-treated animals the responses to further capsaicin applications were almost completely abolished (2.6 ± 1.8 and $1 \pm 0.6\%$; Fig. 1A).

Topical application of acrolein at 300 μ M had similar effects. In control animals three consecutive applications increased meningeal blood flow by 14.4 ± 2.4 , 13.3 ± 3.7 and $8.9 \pm 2.5\%$ ($n = 9$), respectively. These increases in blood flow were significantly reduced in

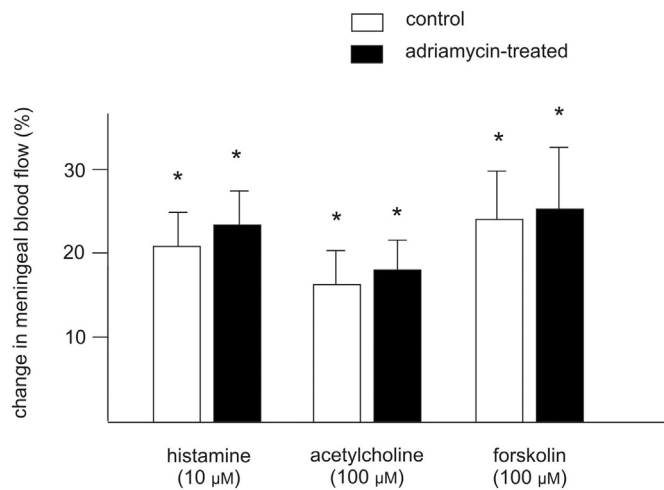


Fig. 2. Effects of dural applications of histamine (10 μM), acetylcholine (100 μM) and forskolin (100 μM) on meningeal blood flow. *: statistically different from the basal flow.

adriamycin-treated animals during all three consecutive applications (4.1 ± 1.1 , 2.6 ± 1.3 and $1.4 \pm 1.8\%$; $n = 14$; Fig. 1B).

CGRP-induced blood flow increases were also reduced in adriamycin-treated animals, while repeated applications did not show a decrement. Flow increases following CGRP at 10 μM were 20.3 ± 5.3 , 21.8 ± 3.9 and $24.7 \pm 5.3\%$ ($n = 15$) in control and 9.8 ± 1.6 , 10 ± 1.9 and $8.6 \pm 1.8\%$ ($n = 17$) in adriamycin-treated animals (Fig. 1C).

In the two groups of animals no significant differences in meningeal blood flow responses were seen following single topical applications of histamine, acetylcholine or forskolin: they were in control vs. adriamycin-treated animals 21.3 ± 3.9 ($n = 10$) vs. $22.3 \pm 4.6\%$ ($n = 16$) after histamine, 15.8 ± 3.7 ($n = 12$) vs. $16.9 \pm 4.5\%$ ($n = 14$) after acetylcholine and 22 ± 8 ($n = 10$) vs. $22.9 \pm 9.8\%$ ($n = 10$) after forskolin applications (Fig. 2).

Systemic blood pressure of adriamycin-treated animals was slightly below the values measured in controls: 98 ± 12 vs. 84 ± 10 mmHg in control vs. adriamycin-treated animals. Drugs administered topically to the dura mater did in no case influence systemic blood pressure.

3.2. Adriamycin treatment modifies CGRP release from meningeal afferents induced by TRPV1 and TRPA1 receptor activation or depolarizing KCl

Basal release of CGRP measured in the *ex vivo* dura mater preparation was 13 ± 2.7 pg/ml in control and 20 ± 1.9 pg/ml in adriamycin-treated rats, indicating a slight but not significant ($p = 0.054$) increase in spontaneous basal CGRP release in adriamycin-treated animals.

In control rats capsaicin at a concentration of 100 nM induced significant increases in CGRP release which were not significantly different upon repeated administrations (294.2 ± 51.6 , 229.5 ± 56.7 and $251.4 \pm 101.4\%$; $n = 7$). In adriamycin-treated animals ($n = 9$) the first capsaicin application induced a significantly higher increase in CGRP release, which was $564.1 \pm 71.2\%$ of the basal release, while further capsaicin-applications failed to increase the release: 117.1 ± 19.9 and $80.1 \pm 12.5\%$ at the second and third applications of capsaicin (Fig. 3A).

In control rats ($n = 6$) TRPA1 receptor activation by acrolein at 300 μM increased CGRP release to 277.2 ± 25.9 , 361.9 ± 50.6 and $385.6 \pm 83.3\%$ of the basal release at the three consecutive applications. While in adriamycin-treated animals CGRP-releasing effect of the first acrolein application was comparable to that of the control ($273.9 \pm 56.2\%$ of the basal release, $n = 6$), the second and third applications of acrolein induced only moderate increases in CGRP

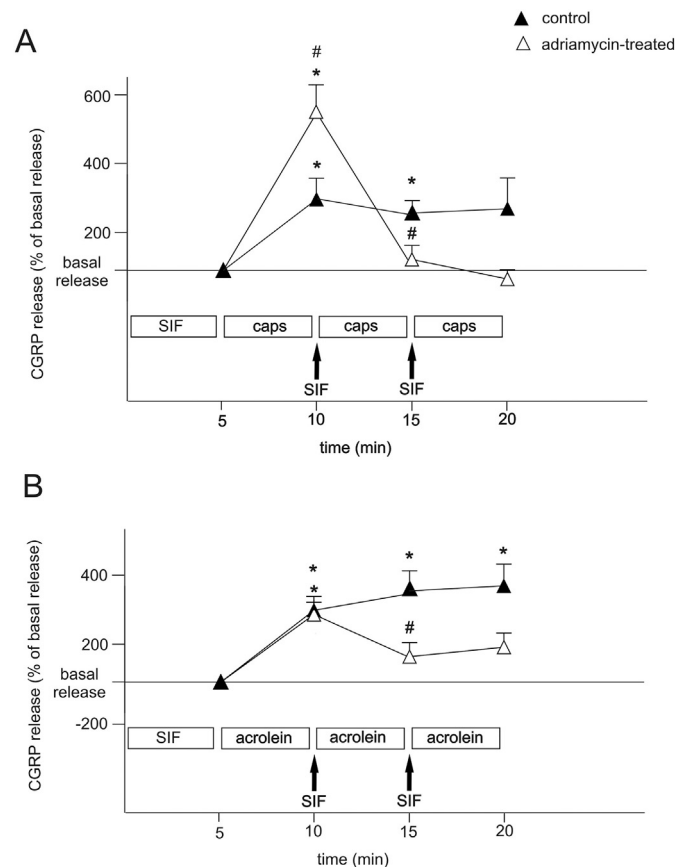


Fig. 3. Capsaicin- (100 nM, A) and acrolein- (300 μM, B) induced release of CGRP from meningeal afferents. CGRP release is expressed as percentage change relative to the basal release. *: statistically different from the basal release, #: statistically different from the control.

release (162.5 ± 40.3 and $189.1 \pm 56.8\%$ of basal release, respectively, Fig. 3B).

Depolarisation of meningeal afferents by KCl (60 mM) increased CGRP release to $141.2 \pm 24\%$ ($n = 7$) and $189.3 \pm 29\%$ ($n = 9$) in control and adriamycin-treated animals, respectively. The difference between adriamycin-treated and control rats was not significant ($p = 0.241$).

3.3. Adriamycin-treatment decreases TRPV1 protein content in the trigeminal ganglion

TRPV1 protein content of trigeminal ganglia obtained from control rats was 6.25 ± 2.7 pg/mg tissue ($n = 10$). Adriamycin-treatment reduced TRPV1 expression to 4 ± 0.5 pg/mg ($n = 11$), although this difference did not reach statistical significance ($p = 0.147$).

3.4. Adriamycin-treatment does not alter TRPV1- and CGRP-immunoreactivity but modifies immunohistochemical staining of CGRP receptor components in the dura mater

In the dura mater of control and adriamycin-treated animals TRPV1- and CGRP-immunoreactive nerve fibers were distributed over the whole supratentorial dura mater. TRPV1- and CGRP-immunoreactive nerves were seen running in nerve bundles parallel with branches of the middle meningeal artery. Single axons were also found in regions distant from larger blood vessels. CGRP was colocalized with TRPV1 in most of these nerve fibers. No obvious difference in the density and distribution of TRPV1- and CGRP-immunoreactive afferents was seen in dura mater preparations of control (Fig. 4A) and adriamycin-treated

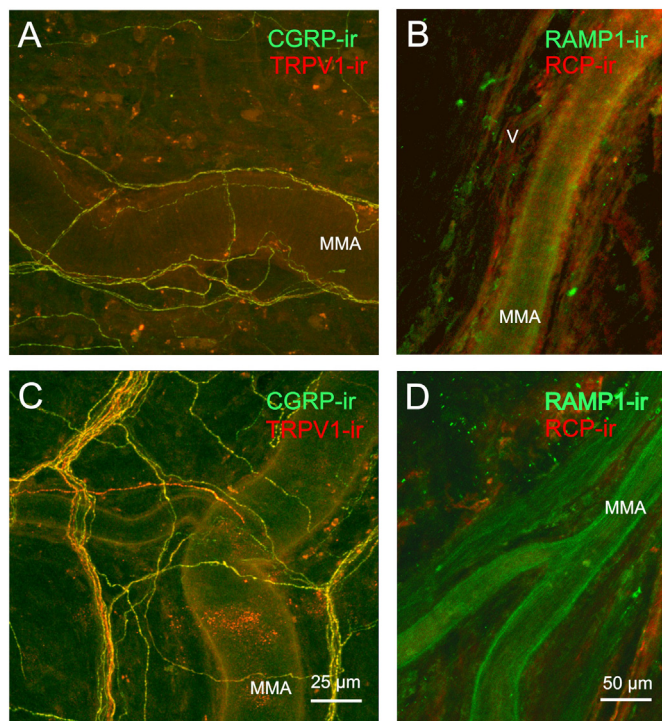


Fig. 4. Photomicrographs showing the distribution of TRPV1-, CGRP- (A, C) and CGRP receptor component RCP- and RAMP1- (B,D) immunoreactivity in the dura mater of control (A, B) and adriamycin-treated (C, D) rats. Scale bar on C applies also for A; scale bar on D applies also for B. MMA: branch of the middle meningeal artery, V: venous vessel.

animals (Fig. 4C).

In control animals the CGRP receptor component proteins RCP and RAMP1 were colocalized in the wall of meningeal arteries and veins (Fig. 4B). In dura samples of adriamycin-treated animals RAMP1-immunoreactivity was present, but no RCP-immunoreactive structures could be identified in the wall of meningeal blood vessels (Fig. 4D).

4. Discussion

The present experiments aimed to study adriamycin-induced changes in meningeal sensory nerve functions. The study revealed a marked impairment of neurogenic blood flow increase resulting from a reduced release of vasodilator neuropeptides in the dura mater upon noxious stimulation. The increase in blood flow is an indirect measure of the vasodilator response following the activation of the nociceptive ion channels TRPV1 and TRPA1 expressed in chemosensitive dural afferents, which seems to be reduced in adriamycin-treated rats. Diminished responses were even more obvious after repeated stimulation. The predominance of CGRP release from sensory nerve fibers in mediating these vasodilator responses is well established (Brain et al., 1985; Kurosawa et al., 1995; Yisarakun et al., 2015). Accordingly, in an established *ex vivo* dura mater preparation, CGRP release was directly determined. In control rats capsaicin and acrolein, specific agonists acting at TRPV1 and TRPA1 receptors, respectively, elicited reproducible releases of CGRP of largely similar magnitudes. In dura mater preparations obtained from rats treated with adriamycin, a strikingly different pattern of CGRP release appeared at repetitive measurements, *i.e.* CGRP release was impaired only after the second and third stimulation period with capsaicin or acrolein, respectively. In adriamycin-treated dura specimens there was even an increase in initial CGRP release after capsaicin application compared to control rats. This is an unexpected finding, since the capsaicin-induced meningeal vasodilator response, which is mainly mediated by CGRP, was markedly

attenuated in adriamycin-treated animals from the beginning. We cannot explain the exact mechanism of the disparate changes observed in the vasodilator response and the CGRP release but the following considerations may be taken into account.

An intracellular/intraterminal increase in calcium ion concentration is essential for the release of peptides from sensory nerves. Calcium influx is caused by the activation of transient receptor potential ion channels, such as the TRPV1 and the TRPA1 channels, and possibly also by opening of other ion channels which are permeable to calcium. Hence, calcium influx and its regulation are of crucial importance in the mechanisms contributing to the control of peptide release from sensory nerves. It is tempting to suggest that adriamycin-induced changes in calcium influx through the nociceptive membrane influence neuropeptide release. This assumption is supported by previous observations showing that administration of adriamycin increased calcium influx in HeLa cells (Dasdia et al., 1979), and similarly calcium influx may be increased in sensory nerves of adriamycin-treated rats, resulting in increased peptide release. Subsequent decreases in CGRP release even beyond control levels may result from a reduced peptide content of sensory nerves due to the excessive first response upon capsaicin administration. Reduced vasodilator capacity was even more obvious after repeated stimulation of TRPV1 and TRPA1 receptors.

These observations prompted us to examine possible alterations in vascular functions that may lead to impaired vasodilatation upon CGRP release in adriamycin-treated animals. We tested the effects of different vasodilator agents applied directly onto the dura mater. We measured the blood flow increasing effects of histamine, acetylcholine and, in particular, exogenous CGRP. Blood flow increases induced by these drugs are independent of the functional condition of meningeal nerves, since they act directly on receptors localized on endothelial and/or smooth muscle cells of dural blood vessels (Brunt et al., 2015; Dux et al., 2002; Holzer, 1998). Histamine- and acetylcholine-induced vasodilatations were unaltered in adriamycin-treated animals while the blood flow increasing effect of CGRP was reduced to about half of that measured in control animals. The apparent similarity of histamine- and acetylcholine-induced vasodilator responses in control and in adriamycin-treated animals suggests that damage to vascular smooth muscle is unlikely to be responsible for the decreased vasodilatation to CGRP. Although earlier observations indicated apoptotic and necrotic changes in vascular smooth muscle after chronic exposure to adriamycin (Murata et al., 2001), our functional results did not suggest a generalized impairment of vascular contractility. In our study we also tested the integrity of intracellular mechanisms involved in CGRP-induced vasodilatation. In adriamycin-treated rats they seemed to be preserved; forskolin, activating adenylyl cyclase and increasing intracellular levels of cAMP (Kanse et al., 1991) elicited similar increases in blood flow in both groups of animals.

Immunohistochemistry revealed a loss of CGRP receptor component RCP-staining in arterial and venous dural blood vessels of adriamycin-treated animals. The CGRP receptor is a multimer made up of a G-protein-coupled receptor called calcitonin-like receptor (CLR), a small transmembrane protein RAMP1 and a cytoplasmic protein RCP (Evans et al., 2000). RCP is considered to enhance receptor coupling to the G-protein signaling machinery. RCP protein expression seems to correlate with CGRP efficacy *in vivo*, suggesting its crucial role in the regulation of CGRP signaling (Dickerson, 2013). RCP protein staining was undetectable in dura samples of adriamycin-treated animals indicating a significant change in protein structure resulting in a loss of immunohistochemical staining and an impairment of CGRP binding of the receptor complex.

Our data indicate multiple but selective impairments of receptor function in the trigeminovascular system after adriamycin treatment. Altered TRPV1 and TRPA1 receptor functions in peripheral afferents results in a robust release of CGRP upon the first application of the corresponding agonist and in a reduced peptide release when the receptor is stimulated repeatedly. Taken together, these data indicate that

the markedly decreased vasodilator responses upon administration of capsaicin and acrolein in adriamycin-pretreated rats may be explained by alterations in CGRP signaling of the vascular smooth muscle. The present findings also suggest that local regulatory/sensory efferent functions of chemosensitive afferent nerves may be profoundly affected by the dynamics of neural peptide release.

While impairment of chemosensitive nociceptors was clearly signalled by decreased TRPV1 protein in the trigeminal ganglia of adriamycin-treated animals, in the dura mater whole mount preparations of control and adriamycin-treated animals the density and distribution of TRPV1- and CGRP-immunoreactive nerves were apparently similar. Despite the known limitations of immunohistochemistry to detect moderate changes in protein content, our findings indicate that reduction of capsaicin- (and probably also acrolein-) induced neurogenic sensory vasodilatation was brought about in the absence of apparent changes in the distribution and localization of TRPV1- and CGRP-immunoreactive dural afferent nerves. We assume that changes in TRPV1 and TRPA1 receptor functions may be similar after adriamycin-treatment, since the TRPA1 receptor is present on chemosensitive neurons that also express TRPV1. Earlier observations in our laboratory indicated that pathophysiological conditions altering the functions of chemosensitive afferents may affect the function of both receptors (Marics et al., 2017; Marics et al., 2016).

A selective impairment of vascular contractility after adriamycin-treatment has been reported earlier. In isolated arteries adriamycin-treatment downregulated the expression of $\alpha 1A$ adrenoceptors but left the expression of endothelin A receptors unaltered (Murata et al., 2001). Anthracycline-induced toxicity is believed to be related to the generation of reactive oxygen species. Indeed, production of superoxide radicals contributed to reduced expression of the $\alpha 1A$ receptor of blood vessels. Also receptors of chemosensitive afferents can be targets of reactive oxygen species. Both TRPV1- and TRPA1 receptors can be modified by these mediators altering the gating properties of the ion channels (Nishio et al., 2013; Ruan et al., 2014).

Besides functional changes of TRPV1 and TRPA1 receptors expressed on chemosensitive afferents, earlier morphological observations in our laboratory also indicated loss of intraepidermal sensory nerve endings but not subepidermal nerve plexuses after adriamycin treatment (Boros et al., 2016). Although our results obtained from dura mater preparations does not indicate loss of TRPV1- or CGRP-immunoreactivity in general, we cannot exclude the possibility of a similar destruction of the most terminal compartments of sensory axons that, however, are hardly detected in preparations of the dura mater due to inherent differences in the organization of cutaneous and dural innervation.

In conclusion, this study demonstrates an impairment of the nociceptive function of peptidergic meningeal afferents that results in diminished sensory neurogenic vasodilatation of meningeal blood vessels in adriamycin-treated rats. The findings suggest that alterations in neuronal CGRP release and changes in RCP receptor complex protein expression may underlie the altered vascular responses. Adriamycin-induced impairment of vascular functions may affect sensory nerve-mediated local tissue reactions and protective mechanisms, such as neurogenic inflammation, which operate in the meninges.

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